When CELL

Investigating solute concentration, osmosis, and cryopreservation

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MOST ANIMALS AVOID THE STRESSES of winter's low temperatures by migrating or seeking protected sites for hibernating, but some insects, frogs, and turtles are freezing tolerant and survive extensive internal ice formation (Storey and Storey, 1990). Currently, a variety of human cells and simple tissues including eggs, sperm, embryos, red blood cells, and heart valves can be successfully cryopreserved; however, there are no methods available for long-term banking of the heart, liver, and other organs. Recent investigations of the mechanisms that help naturally freezing-tolerant vertebrates to survive cold temperatures provide clues for developing protocols for organ cryopreservation (Costanzo et al, 1995).

By studying the topics of freezing and cryopreservation, biology students can gain insight into the critical concepts of diffusion and osmosis. In this article we present information and student activities designed to clarify these concepts by showing how they relate to cryopreservation and natural freezing tolerance in animals.

FREEZING TOLERANCE IN WOOD FROGS

The wood frog, *Rana sylvatica*, is widely distributed in North America from the southern Appalachian Mountains to north of the Arctic Circle in Alaska. This species

freeze over...

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overwinters terrestrially in shallow depressions beneath leaf litter and snow. Adult frogs regularly survive the freezing of as much as 70 percent of their body water for at least several weeks at -20° C. It is generally believed that in order for cells to survive freezing, ice formation must be restricted to extracellular spaces—intracellular freezing is lethal due, in part, to mechanical damage by the ice lattice. When ice forms in extracellular spaces, water molecules join the ice lattice, leaving behind solutes that become progressively more concentrated. As the solute concentration increases outside the cells, intracellular water travels out of the cell, across the cell membrane by osmosis. The excessive dehydration of cells that results is a principal mechanism of freezing injury.

When the wood frog is exposed to freezing temperatures, glycogen stored in its liver is rapidly converted into glucose. Within hours, glucose levels may increase more than 70-fold. Production of these high levels (0.5–1.0 M) of glucose and related compounds, such as glycerol, provides cryoprotection against freezing injury in a number of ways. For example, these substances may directly protect proteins and cell membranes from injury by stabilizing proteins and lipids during cycles of freezing and thawing. Cryoprotectants also act as antifreeze by colligatively depressing the freezing point of body fluids and reducing the amount of ice that forms at a given subzero temperature. This reduction in ice formation limits extracellular solute concentration and cellular dehydration. Furthermore, reducing the amount of ice that forms within organs lessens the chance of mechanical injury to cells from ice crystals.

Current protocols for cryopreserving mammalian cells often rely on a two-step process. In the first part of the procedure, cells are held at relatively high subzero temperatures (above -20°C) as ice forms in the surrounding solution. This step causes the cells to partially dehydrate, minimizing the amount of ice that forms in the second step when the cells are rapidly cooled to -80°C or lower.

THE EFFECTS OF SOLUTES

As temperature decreases, water molecules move more slowly and are chemically attracted to one another, leading to ice crystal formation. Within the ice crystal, each water molecule hydrogen bonds with four others. Energy is required to break these bonds and convert the ice back to water (melting).

To illustrate how solutes can help protect cells from injury during freezing, students carry out the following activity using two film canisters, tap water, two petri dishes, a 50 percent mass/volume solution of table sugar, dark food coloring, a freezer, markers, and tape to label canisters. In one canister, students place 20 mL of tap water and 3 drops of food coloring. In the second canister, students place 20 mL of sugar solution and 3 drops of food coloring. After freezing both canisters overnight, students warm the canisters by holding them in their hands or dipping them in water, thawing them just enough to slide the frozen contents onto petri dishes.

Students write and draw their observations of the shape of the frozen material, the concentration and distribution of the food coloring, and the rate of melting for each sample. Then they explain the differences between the contents of the two canisters. In contrast to the frozen water solution, the frozen sugar solution has a light-colored region of ice from which the sugar and dye molecules have been largely excluded. The dark-colored area of this sample therefore contains a high solute concentration. This concentration of solute lowers the freezing point of the remaining unfrozen water and reduces ice formation; therefore, the cylinder containing the sugar solution melts more quickly because less ice has formed and less heat is required for melting.

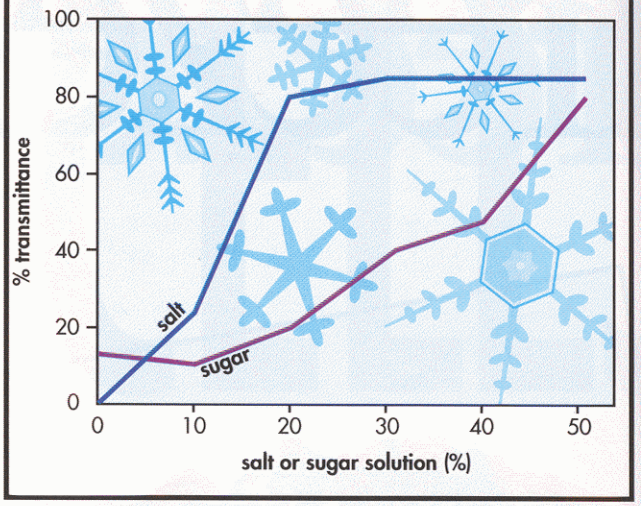
DEHYDRATING CELLS BEFORE FREEZING

If intracellular ice formation is not reduced, cell membranes may rupture and cell contents may leak out. Freezing damage can be easily seen and measured using beets (*Beta vulgaris*), which release their bright red pigment (betacyanin) when frozen (Vodopich and Moore, 1989). Discoloration of potato slices is another way to demonstrate freezing injury.

If cellular dehydration is not excessive, removal of intracellular water increases the capacity of cells to withstand freezing. Cells placed in a hypertonic solution (in which the solute concentration is higher and the relative water concentration is lower than inside the cell) dehydrate by osmosis. The greater the extracellular solute concentration (the more solute particles per unit volume), the more cellular water is lost because water moves osmotically across a semipermeable membrane from regions of lower to higher solute concentration.

FIGURE 1.

The effect of solute concentration on cell damage as measured by betacyanin leakage in frozen beets.



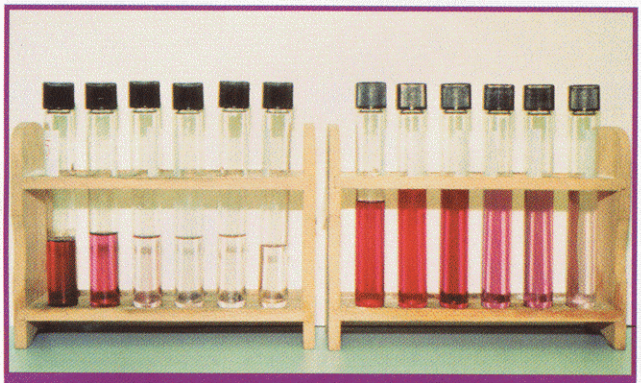
Colligative properties of solutions, such as osmotic pressure and melting point, are determined by the number of particles in solution, not by particle type or size. For example, a sodium ion, a glucose molecule, and a soluble protein would each contribute equally to the solute concentration. Theoretically, a 1.0 M solution of NaCl would produce two moles of osmotically active solute (one mole of sodium ions and one mole of chloride ions). In practice, particularly at higher concentrations, not all molecules completely dissociate, and actual solute concentration is slightly less than predicted.

CRYOPROTECTION IN THE LAB

For this activity, students need a raw beet and a raw potato; a knife; six film canisters; a metric ruler; 0, 10, 20, 30, 40, and 50 percent mass/volume solutions of table salt and sugar; six test tubes; a test tube rack; and a funnel (for those using beets). A spectrophotometer and cuvettes are optional. Students are assigned to four groups as follows—beets in salt solutions, beets in sugar solutions, potatoes in salt solutions, and potatoes in sugar solutions.

Each group prepares the salt or sugar solutions listed above and labels a set of canisters with the concentration, type of solution, and cell type, then cuts the potato or beet into six sections of 2 cm x 1 cm x 0.5 cm each. After rinsing off and patting dry each section, groups place the slices into 20 mL of the assigned salt or sugar solutions and freeze the canisters overnight.

At the beginning of the next class, groups remove the canisters from the freezer. After thawing, the potato slices are placed (in order of increasing solute concentration) on a paper towel; students should pour the liquid from the beet canisters into test tubes in order of increas-



Cellular damage, measured by the release of pigment from beets, decreases as solute concentration increases. Salt (left) was more effective than sugar (right) in reducing cellular damage.

ing solute concentration. Students can then discard the potato liquid and the beet. Students can assess cellular and tissue damage by observing the texture and color of the potato and the color of the beet solution in the test tubes. They should prepare a data table and record their observations. If a spectrophotometer is available, students can use it to measure the percent transmittance of the beet solutions at 460 nm (Figure 1). Groups should graph their data using graph paper, a computer program, or a graphing calculator and share their data with the class. Another method of assessing cell damage is to blot and take the mass of the potato and beet samples before and after freezing to determine the volume of liquid lost during freezing.

After completing the activity, students can interpret their results by responding to the following:

- Which solute was most effective in reducing cellular damage?
- Which concentration(s) were most effective in reducing cellular damage?
- Describe a solution that could be used to rehydrate these cells during thawing.
- Explain why there is a difference between salt and sugar treatments.
- Is there any evidence that these cells are viable?
- As a researcher, you may be interested in more than the release of pigments from beet cells, which contain other organic molecules, such as sugars. Predict the results of a test for the presence of sugars released from beet cells in the 0 and 50 percent salt solutions. If possible, test your predictions using Benedict's solution.

To further assess this activity, teachers can give students additional projects and questions related to the topics of osmosis and freezing. For example:

- Design an economical cryopreservation procedure for beet cells.
- Based on the results of the sugar and salt experiments, evaluate the effectiveness of $CaCl_2$ as a cryoprotectant.
- Explain why many homemade popsicles have an area that is darker-colored and sweeter than the rest of the popsicle.
- Why is salt placed on roads and sidewalks when the temperature nears the freezing point?
- Predict the effect of salt on plants that grow on roadsides.
- Why is antifreeze added to car radiators?

UNDERSTANDING OSMOSIS

Living cells can be cryopreserved by treating them prior to freezing with a hypertonic solution that increases cell dehydration and thereby decreases cellular damage. The concentration of solute particles in solution determines the amount of cellular dehydration. In these experiments, salt was a more effective cryoprotectant than sugar because the salt crystals dissociated in water, effectively doubling the concentration of solutes in solution. Upon thawing, cells can be rehydrated by placing them in a hypotonic solution.

Once tenth grade biology students have observed the effects of solutes on cell dehydration, they can apply their experiences and achieve a better understanding of protocols for embryo and organ cryopreservation and adaptations for natural freezing tolerance in animals. Because the students were focused on an application of osmosis, they began to understand the role of solutes as the determining factor for osmotic movement of water. These activities can also provide a basis for more advanced students to review, use, and apply chemistry knowledge in novel ways. ✧

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